INNOVATIVE POTENTIAL™

A report for Innovative Potential on the unpublished findings of:

A methodology to assess the ecological integration of terrestrial invasive plant species in soil ecosystems.

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Abstract

This document was prepared for Innovative Potential regarding unpublished work conducted during the 2014-2015 Northern Ontario Heritage Fund Corporation internship at the Antunes Soil Science and Ecology laboratory. The original manuscript and experimentation found was to assess the relative integration of an invasive plant species with host soil ecologies using a low-technology threshold root-survey framework to make the technique easily and more widely adoptable by formal laboratories and citizen science programs. In total ten (10) invasive plant species from across North America were surveyed two (2) of which (*Bromus inermis and Leucanthemum vulgare*) were grown under laboratory and field conditions as controls. Evidence for common soil microorganism interactions (lesions) with host roots was assessed using a light-microscope. A systematic field-of-vision approach was used to tally observations of mycorrhizal fungi (MF), dark septate endophytes (DSE), herbivory, non-descript tissue damage (injury), single cell damage (decay), and unidentified cellular structures and organisms (other). The results of this study found significance differences between species, treatments, and lesion accumulation profiles.

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Foreword

The structure of this document for Innovative Potential is the original manuscript written in 2015 by Jordache Boudreau, with commentary interjections with insights for Innovative Potential. This format is used to preserve the original work and intents, as well as to draw attention and comparison to the decade and contexts that have changed from 2015-2025, the original manuscript is unchanged and unedited. Pedro Antunes is acknowledged in providing funding for the project through the Terrestrially Invasive Plant Species (TIPS) Network, and a Northern Ontario Heritage Fund Corporation internship; however: Pedro did not play a role in experimental design and or writing the original manuscript, that work was done by Jordache Boudreau from 2014-2015. The document is basically the original manuscript as is, with a comment section being introduced at the end of the discussion section for Innovative Potential (see: section 4.1.9).

1.1 Introduction

Ecology has long sought to understand the mechanisms responsible for the invasion and success of exotic plant species. Currently, one of the main hypotheses surrounding the explanation of successful plant invasions is the release of exotic invasive plants from natural enemies in their introduced range, giving them a competitive advantage. Although studies involving home/away comparisons have demonstrated that exotic invasive species do leave behind natural enemies, this does not mean that they are completely free from native enemies. For example, there are still many interactions between the non-native plant and more generalist soil pathogens in their introduced range. However, it has been shown that non-native plants do not accumulate pathogens as fast as native plants do, suggesting that it is the absence of specialist pathogens that may be a key driver of plant invasions.

Although plant-soil feedback experiments conducted in the greenhouse revealed a difference between natives and exotic invasives in their susceptibility to pathogen damage, it is possible that specialist enemies have started to co-adapt with invasive exotics, particularly in the areas where they were first introduced. Until recently, evolutionary change was not considered in invasion ecology because it was assumed that evolutionary processes were slow. However, we now have evidence that supports a very fast rate of adaptive evolution in many areas of the life sciences. With this project we aimed to investigate whether pathogens and other enemies (e.g., mesofauna and below-ground insect herbivores) have started to ecologically accumulate and specialize in exotic invasive plant species.

While there have been thousands of introduced species brought to North America after the European colonization, only a very small percentage of these species become invasive due to reasons which are entirely unknown at this time. There are several theories which have emerged attempting to explain the invasion phenomenon. One such theory, the predator release hypothesis (PEH), states that invasive plants are invasive because of an absence, or release, of a suppressive factor from their native habitats. Freed of suppressive constraints; and more importantly, absent those in the new environment, the introduced species is now only limited by its ability to proliferate, spread, and the local growing season. A consideration

of escaped suppressive factors can include predation; regularly occurring climatic or ecological events (e.g., monsoons, or insect swarms); and diseases.

A majority of the research over the last thirty years regarding terrestrially invasive plant species (invasive species; TIPS) has focused on aspects of the plants biology; the effects of ecological damage caused by its invasion; and management and eradication efforts. Most of this body of research has concerned itself with the above-ground biological and ecological functions of the plant, with a relative neglect of consideration to the living soils and root systems which support these invasive plants. Of the research involving invasive plant species and soil, most have focused on the physical aspects (e.g., salinity, mineral composition, acidity, etc.); however, the role of the soil ecosystem has yet to be characterized in the context of invasive plant species.

For example, one of the most well-known groups of soil organisms are the mycorrhyzal fungi (MF), a globally distributed symbiotic family primarily responsible for scavenging phosphorus and nitrates in exchange for carbohydrates from the host plant. Estimated to form relationships with 80% of all of the plant species on Earth, the role MF play in a species invasion is poorly understood; in part due to the lack of research efforts. Although the majority of these fungi are not host specific, the hyphae networks are known to form between individual plants of different species, and there is a yet-undetermined selection process that occurs where one host species is preferred over another. Whether or not invasive species as a whole are preferred to native hosts is not yet known.

What also is not known is the role soil-resident pathogenic microorganisms play in maintaining invasive and native populations. The assumption under the PEH is essentially that a native ecosystem is adapted to its own diseases, and that these diseases are at a tentative equilibrium with host plant populations. The invasive species on the other hand proliferates; as it is unrecognized by these native pathogens, the result being the continuing range expansion and the destruction of the native species habitat. Over decades or centuries, it is hypothesized that the resident pathogens will be able to recognize the invasive plant; and, having found a new host, be able to crash or curb the invasive population.

In an effort to better understand the integration between invasive species and native soil ecosystems, a generalized and systematic observational approach was developed. The methodology described in this paper is intended as a broad approach aimed at assessing and directly comparing invasive and native species both with each other and themselves. Similar approaches have been used to characterize mycorrhizal fungi and other soil microorganisms, however, there has yet to be described a standard method. To do this, a large-sample root survey of eight highly invasive species across North America was conducted, and six ecologically ubiquitous categories were obtained; i.e., those which were in common with each of the species. To verify the applicability of these categories, two additional invasive species to North America were selected (*Bromus inermis* and *Leucanthemum vulgare*), and the method was used on both laboratory and field-grown samples. The end result is a simple and accessible methodology for gaining insight into the integration of invasive plant species and local soil ecologies.

We formally introduce the qualitative definition of lesions to denote any aberration to the pristine root condition caused by any of the diverse root-soil-microbe interactions observed throughout this methodology.

2.1 Materials and Methods

2.1.1 Root sampling and preservation

Root samples (<2.0 mm diameter) of nine invasive plant species were collected by volunteers from across North America during the summer of 2014 (n=165; supplemental figure 1). Species included what are considered highly invasive and nuisance species such as garlic mustard (*Alliaria petioloata*); cheatgrass (*Bromus tectorum*); spotted knapweed (*Centaurea stoebe*); Japanese knotweed (*Fallopia japonica*); purple loosestrife (*Lythrum salicaria*); Japanese stiltgrass (*Microstegium vimineum*); European buckthorn (*Rhamnus cathartica*); saltcedar (*Tamarix ramisissima*); and dog-strangling vine (*Vincetoxicum rossicum*). Root samples that were collected from the field were preserved by desiccation in a drying oven at 60^{oC} for three days. Data detailing the plant population density, area of infestation, location, plant height, land use, and damage estimates were also collected during sampling.

2.1.2 Root clearing and staining

Root segments (<2.0 mm diameter) were arbitrarily selected from dried samples and placed into tissue cassettes (HistoPrep® OmniSette® by Fisher Scientific; Pittsburgh, PA). Samples were rehydrated for 15 minutes in deionized (DI) water; then, cassettes were placed into a 10% (w/v) KOH (Fisher Scientific; Fair Lawn, NJ) solution in an 80^{o}C water bath for one hour to clear. For some species (*L. salicara*; *T. ramosissima*; *R. Cathartica*; and *F. japonica*) samples required bleaching with hydrogen peroxide; this was performed after treatment in the 10% KOH solution. Samples requiring bleaching were soaked overnight (approximately 8-10 hours) in a 1.0 L solution of 150 ml 3% hydrogen peroxide (Fisher Scientific; Fair Lawn, NJ) and 5.0 ml ammonium hydroxide (Acros, New Jersey, USA). To stain, the cassettes were rinsed with DI water before being placed in 1% (v/v) HCl (Fisher Scientific; Fair Lawn, NJ) for 15 minutes in the water bath. the acid was decanted and the volume was replaced with a 0.05% (w/v) solution of Trypan blue dye (Fisher Scientific; Fair Lawn, NJ). The cassettes were placed into an 80^{o}C water bath for 20 minutes. The dye was then decanted and the cassettes were left to destain overnight in a 50% glycerol-water solution (Fisher Scientific; Fair Lawn, NJ). Roots in the destain could be left for several weeks before being mounted onto microscope slides. Please see the supplemental material for protocol modifications for each individual species. Stained root segments were then mounted on microscope slides, and a drop of 50% glycerol was added for hydration and preservation. The slides were sealed underneath a 20 x 50 mm cover slip and sealed with commercial clear-coat nail polish (Rimmel London, SalonPRO; New York, NY).

2.1.3 Root category classification and quantification

Prepared slides were assessed in a fashion similar to McGonigle *et al.* (1993); that is, in a linear and systematic fashion (Supplemental figure X). However, a modification was required for counting lesions due to the variance in their physical sizes (e.g., multi-cellular versus intracellular sizes); this modification is the abandonment of McGonigle et al.'s crosshairs in favor of a field of view (FOV) assessment. For each slide prepared, a minimum of 100 root intersections were counted to overcome observer bias (McGonigle *et al,.* 1993). For each

observation of root segment (the intersection), the lesions present in the entire FOV were noted. As depicted in Supplemental Figure 1, the microscope stage is moved perpendicularly across the alignment of the roots. As such, the risk of double counting the same lesions is reduced.

2.1.4 Categorizing lesions

Root samples from each of the above-mentioned plant species were examined microscopically (as described above) and a tally was kept of the various microorganisms, physical injuries, and staining bodies observed. The observations with the highest frequency across species were selected as candidate categories for further development and characterization as part of the general methodology described in this paper. The most common categories of lesions shared by each of these invasive plant species were mycorrhizal fungi (MF); dark septated endophytes (DSE); unidentified cellular structures and organisms (i.e., miscellaneous or other; O); herbivorous chew marks (HE); single cell damage or decay (DC); and non-descript tissue damage or a general injury (IN); Please see results section for a discussion of each lesion category.

2.1.5 Effects of lesions on plant growth

A completely randomized block design (CRBD) of six treatments with six replicates (n=36) and two different invasive species (*Leucanthemum vulgare* Lam. (syn. *Chysanthemum leucanthemum* L., "ox-eye daisy"; American Meadows Seed Company, Williston, VT, USA)) and *Bromus inermis sp. inermis* Leyss., "smooth brome"; OFRI arboretum) was used to investigate the effects of soil sieving on lesion category accumulation and distribution. Treatments consisted of field soil sieved at 2000 µm, 600 µm, 38 µm, and through a No. 1 Whatman filter paper; controls consisted of unsieved sterilized soil (negative control; Sterile; ST) and unsieved unsterilized soil (positive control; Field Soil; FS). Field soil was collected from Algoma University's long-term research field site at the Ontario Forestry Research Institute (OFRI) arboretum in Sault Ste. Marie, ON, Canada in August 2014 (N 46°32.5749, W 84°27.5439). The silty loam soil at this location was previously characterized to have a pH of 5.8 and contained 0.13% total N; 1.81% total C; 11.4 mg plant extractable P kg^{-1}; 360.7 mg Ca kg^{-1}; 48.9 mg K kg^{-1}; and 38.9 Mg kg^{-1}. Field soil was autoclaved for four (4) hours at 121^{oC} in a vacuum cycle and stored at room temperature in sealed autoclave bags for 14 days to abate the nutrient flush. The sterilized soil was mixed in a 2:1 ratio with Turface to act as a bulking substrate. Inoculated treatments were prepared by passing an aqueous suspension of field soil through a respectively sized sieve, and mixing the collected filtrate into the sterile soil/Turface bulk (250ml kg^{-1}). Due to the convenience of the sieve size, the raw, 2000 µm sieve, and sterile treatments were potted directly without the use of Turface. Containers used in the experiment were 164 ml Ray Leach Cone-tainers™ (SC10-R model) that were washed then sterilized in 10% bleach for 30 minutes before being filled with soil treatments. The experiment was carried out in a plant growth chamber set to 55% relative humidity, 16 hours of light at an intensity of 130 mmol m^{-2} s^{-1} at 23^{oC} and eight hours of dark at 20^{oC}. Containers were watered each day with 25 ml deionized water. Plants were

harvested at 12 weeks growth time; stems were cut at soil level and the plants were dried at 60^{oC} for 3 days. Roots were washed and preserved in 50% ethanol before mounting.

2.1.7 Study organisms

Seeds for *L. vulgare* were purchased from the American Meadows Seed Company (Williston, VT, USA) in November of 2010 and stored at 4^{oC} until germinated. *Bromus inermis* seeds were collected from the Invasive Species Research Institute's long-term research field site at the Ontario Forestry Research Institute arboretum in Sault Ste. Marie, ON, Canada in September 2013 (N 46°32.5749, W 84°27.5439); seeds were stored and Seeds were germinated under the same conditions as *L. vulgare* in moist vermiculite.

2.1.8 Data Analysis

The significant differences in the CRBD experiment were analyzed by ANOVA with a post-hoc analysis using Tukey's honest significance test ($p < 0.05$) where applicable. Statistical software used to run analyses was obtained from the R project (r-project.org) and was conducted in the 3.0.1 version.

3.0 Results

3.1.1 Lesion survey

We conducted an exploratory survey of the root systems of the invasive and nuisance species garlic mustard (*Alliaria petioloata*); cheatgrass (*Bromus tectorum*); spotted knapweed (*Centaurea stoebe*); Japanese knotweed (*Fallopia japonica*); purple loosestrife (*Lythrum salicaria*); Japanese stiltgrass (*Microstegium vimineum*); European buckthorn (*Rhamnus cathartica*); saltcedar (*Tamarix ramisissima*); and dog-strangling vine (*Vincetoxicum rossicum*). The purpose of this survey was to ascertain which categorical labels would best be suited for developing a generalized survey protocol for assessing root lesions and quantifying root-soil ecosystem interactions. When root samples were examined microscopically, it was only in the outerlayers of the root (epithelial and cortext cells) in which lesions were observed.

Roots which were typically larger than 2 mm in diameter saw a marked decline in the frequency of lesion observations. Roots with a greater diameter than 2 mm were observationally associated with greater amounts of lignifications; the use of these roots resulted in increased KOH exposure times which would cause epithelial and cortex cells to sough off leaving only the stele. Roots with a physical diameter of less than 2 mm became standard as they were also small enough to be unobstructed three-dimensionally through the entirety of the microscopic plane of focus. Additionally, finer roots (i.e., less lignified) were generally more convenient to work with, required less exposure time to KOH, and were more apt to uniformly stain with the trypan blue dye.

Those lesion categories which were most frequently observed in and across all of the species surveyed were mycorrhizal fungi (MF); dark septate endophytes (DSE); herbivorous chew marks (HE); injury or bruising (IN); single cell decay (DE); and a miscellaneous category, other (O). As an approximate measure of the amount of root sample unoccupied by lesions, root sections with no observations were also counted under the category of none (N; Table 1; Figure 1). Table 1 displays an example for the organizational structure for counting root lesions, it includes multiple root lesions and multiple species; however, it is recommend that only one species be counted at a time to reduce error and confusion.

Table 1. Survery of Five (5) Invasive Plant Species's Root Lesions

Species	Sample	Intersection	Hyphae	Fungi	Herbivory	Injury	Decay	Other	None	Lesion Sum	Lesion %
A	1	89	22	19	3	11	20	0	25	53	60
B	2	115	103	67	0	0	0	3	12	173	150
C	3	68	34	33	3	2	15	0	9	53	78
D	4	56	22	31	4	1	6	1	16	43	77
E	5	111	40	38	0	5	17	15	40	75	68
	SUM	439	221	188	10	19	58	19	102	397	
	AVG	88	44	38	2	4	12	4	20	79	86
	STDEV	26	34	18	2	4	8	6	13	54	37

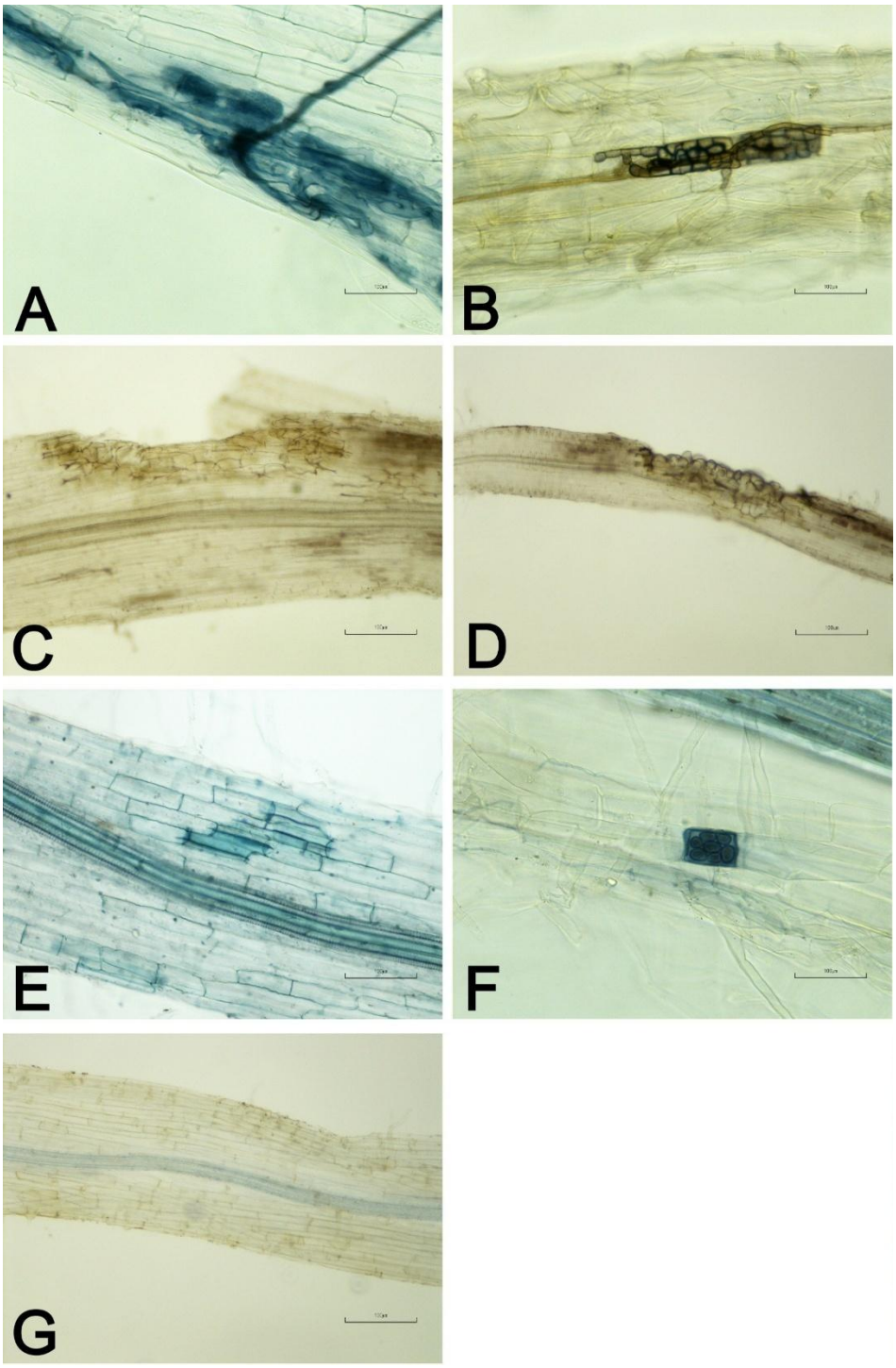

Figure 1. Most frequently observed common plant root-lesion categories across different invasive plant species, different plant species shown. A: mycorrhyzial fungi (MF); B: dark septate endophytes (DSE); C: herbivory (HE); D: injury and or bruising (IN); E: decay (DE); F: other and or miscellaneous (O); G: none (N). Bar: 100 µm.

3.1.2 Lesion Categories

The lesion counting method developed should be considered as a binary count approach (i.e., either a lesion is present or it is not), with the frequency of a given lesion category outlining a plant species' particular root-soil interactions. When counting lesions, all associated structures were counted only once per field of vision.

Consistent distinction between the extracellular hyphae of MF and DSE species was made convenient through the use of trypan blue staining; wherein MF and associated structures (e.g., arbuscules, hyphae, and vesicles) would stain blue, and whereas DSE did not stain, remaining brown. Herbivory, (HE) as a lesion, was easily identifiable. HE lesions presented consistently as an intensely-stained area of root epithelia surrounding a characteristic circular removal of tissue; with the circular centre often penetrating into the cortex layer below (figure 1). However, the stained area surrounding the circular incision would vary in size depending on plant species. Similarly to HE, the injury (IN) lesion would either present as a large (≥150 µm) area of relatively darker-stained epithelia, and/or exhibit cellular or structural damage along the length of the root (figure 1). Typically, the IN lesions did not penetrate below the epithelium layer of the root and lacked the characteristic circular incision seen in HE lesions. The lesion decay (DE) was identified as a single cell or grouping of cells, typically less than five, with distinct and intensely-stained borders (figure 1). The DE lesion was also counted as a singular intensely staining cell. The DE lesion was distinguished from the IN lesion in that DE lesions were comparatively limited in area as compared with IN (figure 1). Finally, the category other (O) was tallied as a catch-all category wherein any microorganism, root process (e.g., starch granules; secretions), or anomali could be counted (figure 2).

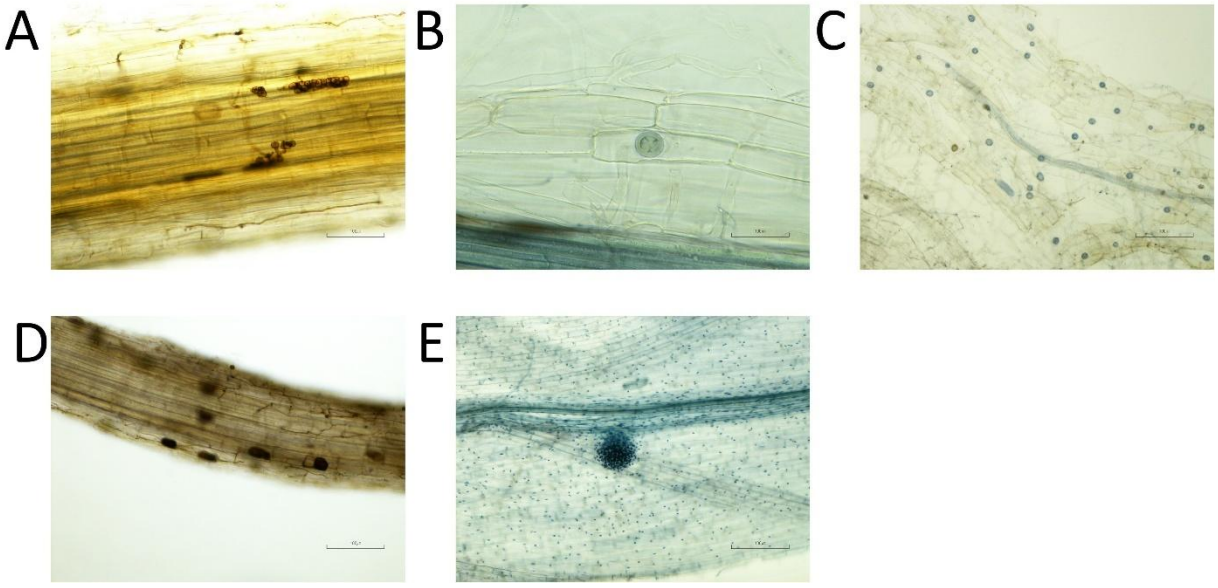

Figure 2. Examples of unidentified plant root anomalies categorized in the Other category. A-D: Unidentified phenomenon; E: Root start. A, D: *B. inermis*; B, C, and E: *L. vulgare*.

3.1.3 Lesion Characterization

In an effort to identify potential sources of histological artifacts introduced during sample processing, *L. vulgare* and *B. inermis* roots were grown in sterilized soil and assessed. Typical artifacts encountered were dark staining bodies (uncleared xylem and phloem); transiently stained roots; torn or removed root epithelia; and tweezer marks. Additionally, newly forming root bifurcations would resemble herbivorous lesions dependent on root orientation, and could be routinely distinguished by the lack of periphery staining and abundant clustering of immature cells. Prolonged exposure to KOH treatment would cause root epithelia to slough, and in some cases (*A. petiolata*) the roots would become gel-like with over exposure. DE and IN lesions observed in these controls were due to normal physiological processes such as root turnover, with the frequency of these observations being rare (<95% of observation). Figure 3 shows how root lesions appeared across the ten (10) species selected for study (see 2.1.1 and 2.1.5).

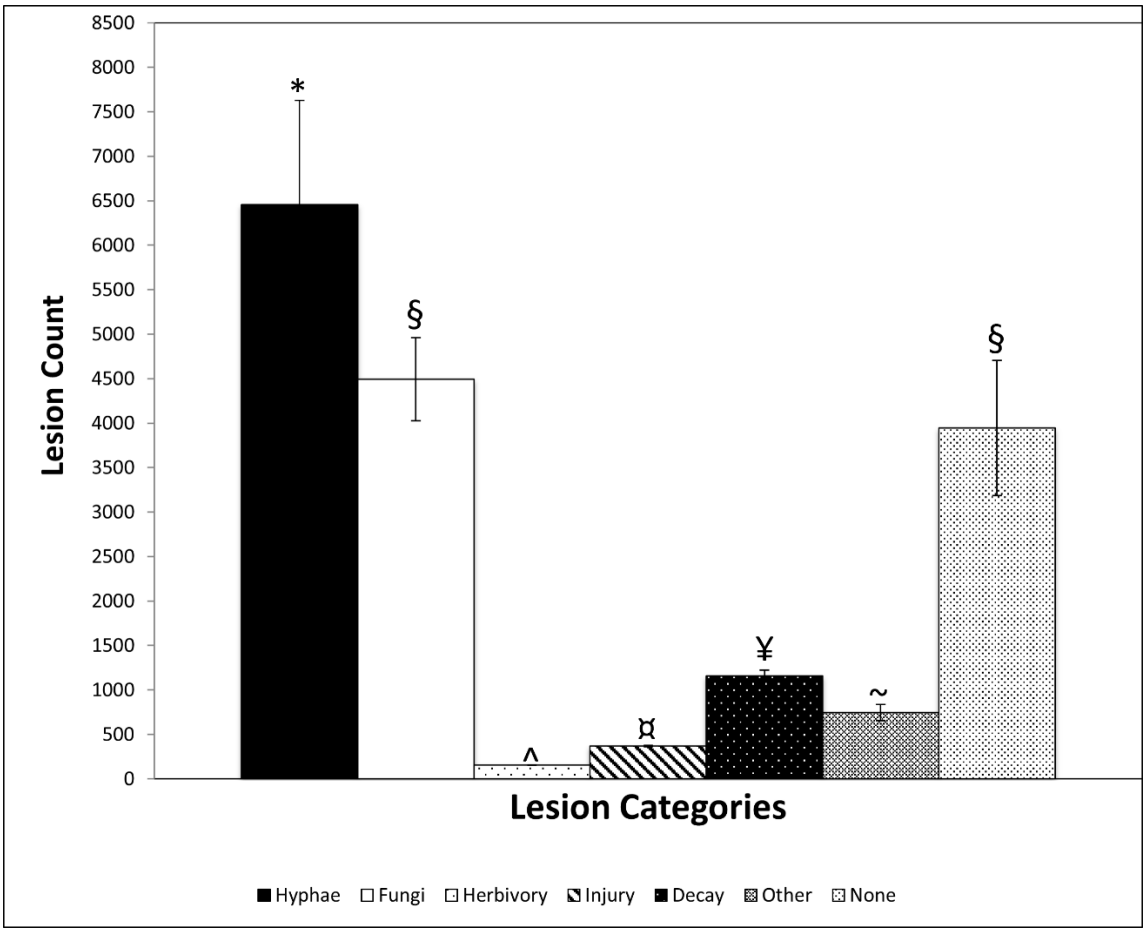

Figure 3. Lesion category counts across 10 grouped and undifferentiated plant species. *, §, ^, ¤, ¥, ~: statistically significant ($p < 0.05$) CRBD ANOVA.

3.2.1 Effects of soil biota on plant biomass

Aboveground plant biomass and root lesion data were collected for *Bromus inermis* and *Leucanthemum vulgare* grown for 12 weeks indoors under conditions described in Section 2.1.5. Treatments consisted of field soil inoculum sieved at 2000 µm; 600 µm; 38 µm; and passed through a Whatman No. 1 filter paper (FP). Control groups in this experiment were comprised of sterilized (ST) and unsterilized (US) field soil. Sterility of the ST control group was verified observationally upon microscopic assessment of the ST treatment group in each replicate and species (i.e., an absence of observable lesions). The dried biomass of both *B. inermis* and *L. vulgare* was found to be directly affected by sieve treatments. The general observation was that as the degree of sterility increased so too did the plant's biomass; i.e., the microorganisms present in the soils suppressed plant growth (Figure 4).

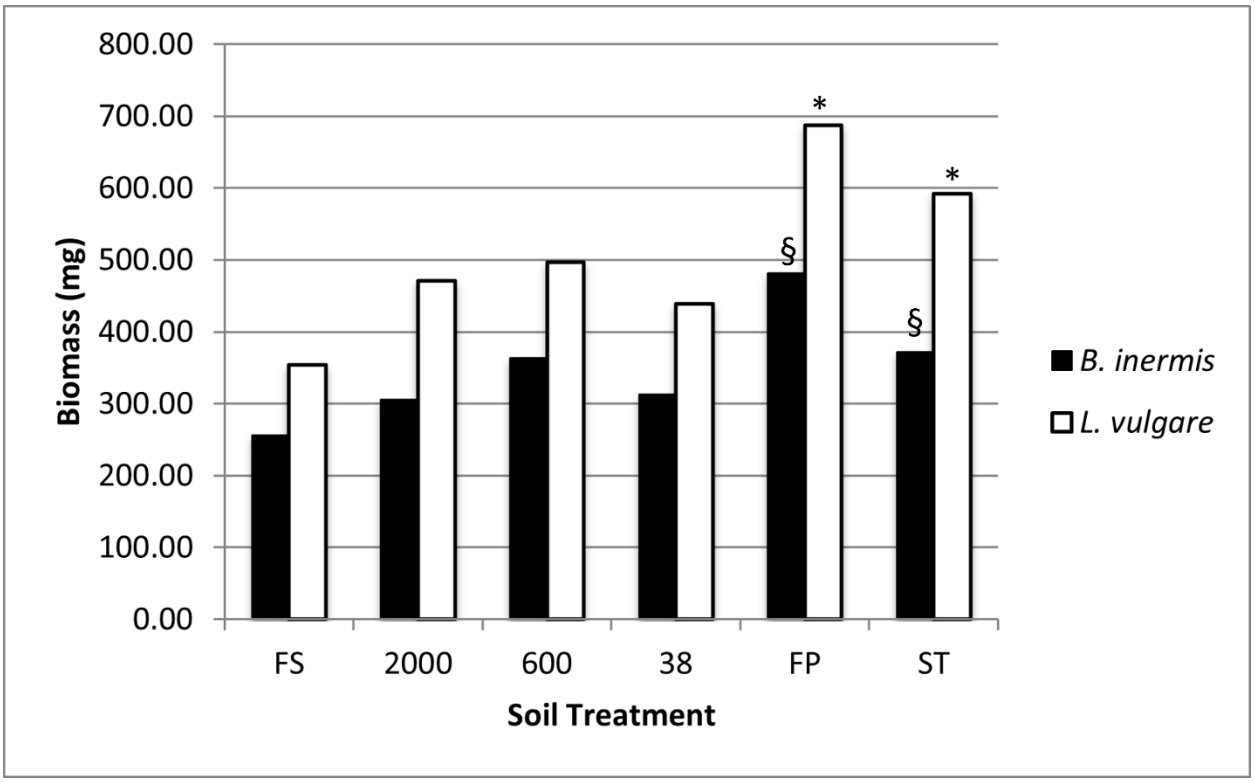

Figure 4. Comparison of plant biomass (mg) of *B. inermis* and *L. vulgare* in plants grown in soil sieve size treatments. FS: field soil; 2000: 2000 um mesh size; 600 um mesh size; 38 um mesh size; FP: Whatman No. 1 filter paper; ST: sterilized (autoclaved). *, §: statistically significant ($p < 0.05$) CRBD ANOVA versus FS treatment.

3.2.2 Lesion effects on biomass

Statistically and observationally, there was a significant difference between the gross number of lesions seen per treatment between *B. inermis* and *L. vulgare*. There is also a notable species difference on the number of accumulated lesions based on treatment; *L. vulgare* had more lesions on average per intersection. However, there was no effect of lesion count

on biomass, nor was there an effect of sieve size (treatment) on lesions biomass for either *L. vulgare* or *B. inermis*. There was no co-variance between biomass and lesions based on sieve size.

In counting the frequency of no lesion observations, approximately 30% of the root system of *B. inermis* was affected by root lesions in the unsterilized (US) field soil treatment; *L. vulgare*, in contrast, had a 60% lesion-affected root system in the US treatment. In both species (*B. inermis; L. vulgare*) and in all treatments mycorryhzal fungi (MF) were the predominant observable lesion, followed by dark septate endophytes (DSE). With *B. inermis,* observances of herbivory, injury and decay type lesions were rare (<5%) across all treatments. The observance of other (O) category lesions was approximately 10% in the US, 2000 µm, and 600 µm treatments; no other-type lesions were observed below 600 µm sieve sizes. In the *L. vulgare* experiment, MF lesions ranked highest in observances; however, there was a notable decrease in the observations of DSE. The *L. vulgare* observations of herbivory, injury, decay, and other type lesions were rare (<5%) across all treatments.

3.2.3 Laboratory and Field differences

To understand the differences between field and laboratory grown roots, samples of *B. inermis* and *L. vulgare* were collected from the Ontario Forestry Research Institute (OFRI) arboretum and assessed for lesions. The unsterilized field soil (FS) treatment and the arboretum (ARB) collected samples had no statistical differences between observations of mycorrhizal fungi (MF), Bruising (BR), decay (DE), or other (O) lesion categories for either *B. inermis* or *L. vulgare* (Figure 5). Interestingly, the DSE category for both species of plants had elevated counts in the ARB sample compared to the US treatment.

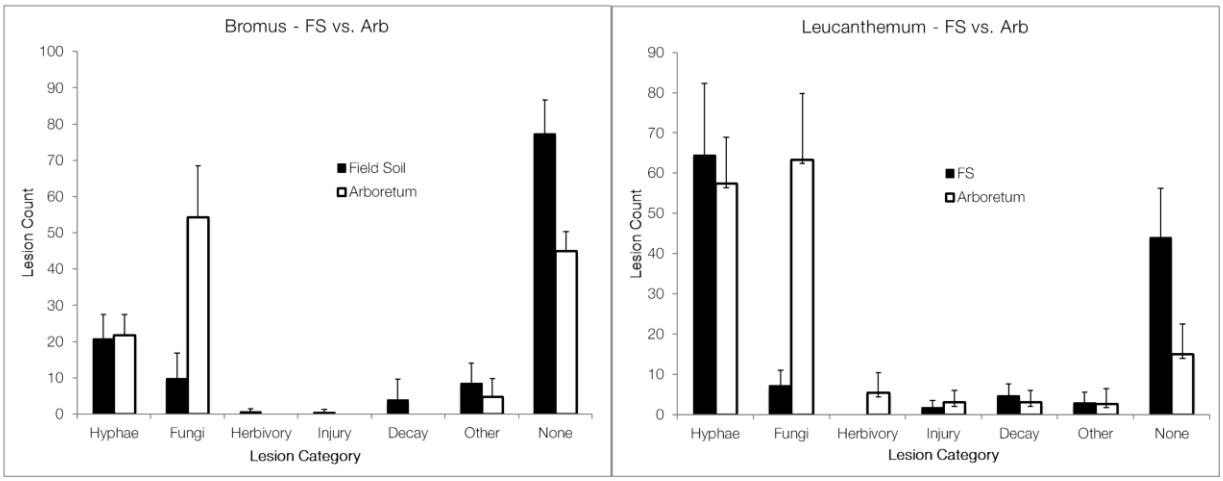

Figure 5. Comparison of root lesions between laboratory-grown and arboretum-grown *B. inermis* and *L. vulgare*. Statistically significant differences found at ($p < 0.05$) CRBD ANOVA for arboretum-grown hyphae lesion counts, and no lesion counts in *B. inermis* (Bromus) and *L. vulgare* (Leucanthemum).

3.2.4 Native and exotic species

Two species native to North America, *Symphyotrichum lanceolatum* (syn. *Aster lanceolatus*) and *Erigeron strigosus,* were collected from the OFRI arboretum and examined for lesions. In a comparison of the pooled lesions between the arboretum-collected natives (*S. Lanceolatum; E. strigosus*) and exotics (*B. inermis; L. vulgare*), there was found to be no statistical differences between exotics versus natives across lesion categories (Figure 6). However, there was a statistically significant difference between natives and exotics in the amount of root unaffected by lesions, with exotics having a higher percentage of roots unaffected that natives. Unpooled analysis shows that his effect is seen primarily due to the inclusion of *B. inermis*. There is an observed and statistically significant behavior ($p < 0.05$) in the Hyphae lesion category in figure 6 wherein an additional co-variable defining plant species is suspected to exist between certain forms of exotics and native plant species (*B. inermis* + *S. lanceolatum*, versus: *L. vulgare* + *E. strigosis*).

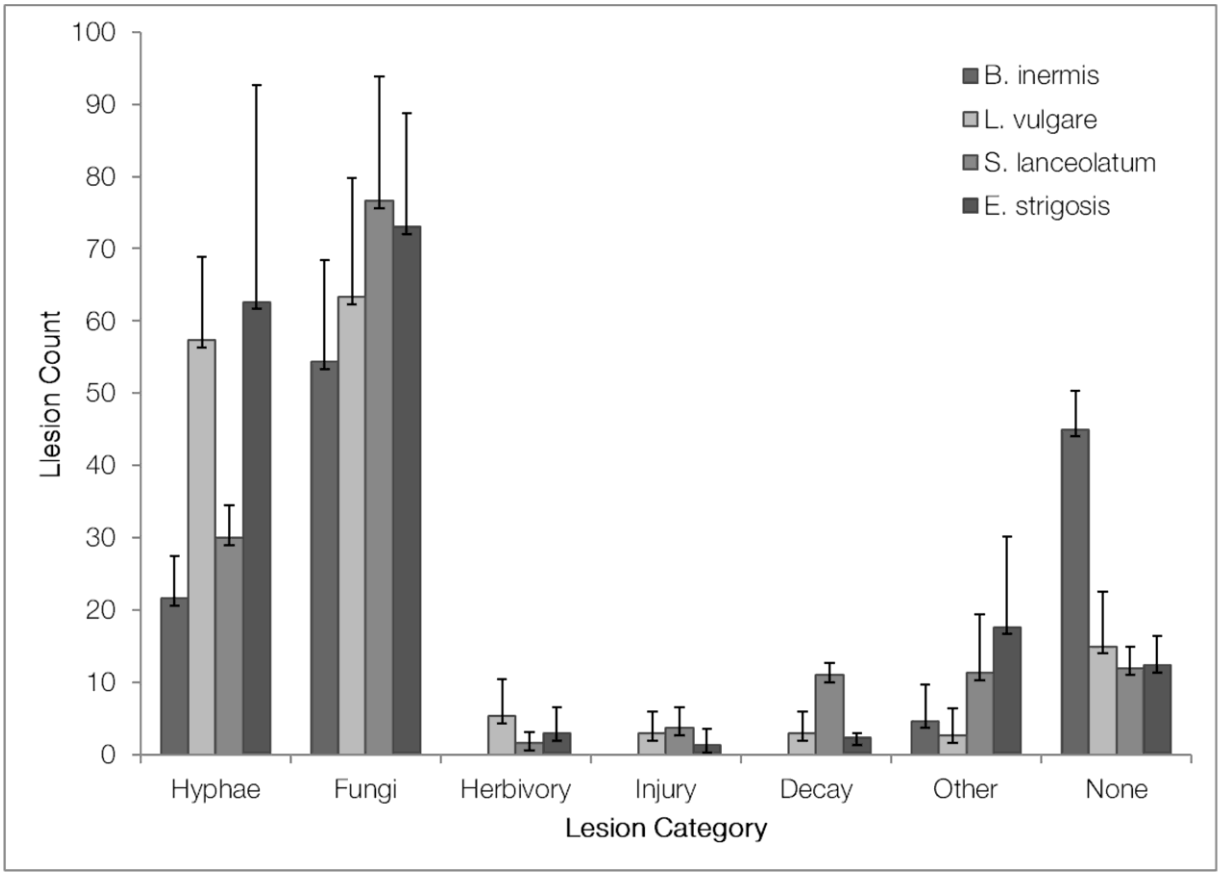

Figure 6. Comparison of root lesions between arboretum-grown invasive plant species *B. inermis* and *L. vulgare*, and native species *S. lanceolatum* and *E. strigosis*. Statistically significant differences found at ($p < 0.05$) CRBD ANOVA for lesion-category differences, but not for plant exotic vs native differences.

4.0 Discussion

4.1.1 Lesion Survey

Roots which were typically larger than 2 mm in diameter saw a marked decline in the frequency of lesion observations, this is believed to occur for two general reasons i) as root diameter increases so too does lignification; and ii) there exists a higher surface area to volume ratio for nutrient abundance in smaller root diameters. Understandably, in roots with higher nutrient exchange capabilities (surface area-to-volume ratio) and greater ease of penetration (less lignification), an increase in gross abundance of soil microorganisms would exist; this observation was seen both within and across all species sampled.

Invasive plant species from several taxa were included in the root survey to ensure a robustness in the methodology, and to ensure that any criterion developed could be applied to any plant species, invasive or otherwise. Attempting to develop a lesion survey methodology built from of a majority of invasive grasses for instance would have been ecologically disingenuous. The robustness of this approach is that it can be used to assess a particular species integration with the surrounding soil ecology.

No attempts were made to identify soil microbiota beyond the categorical distinctions established because the field samples received contained a large degree of unknowable geographic diversity in terms of soil community species compositions. To be able to draw systematic information from such statistical noise is the intent of this methodology; and to do this a broad and general approach was taken.

4.1.2 Lesion Categories

The lesion categories chosen of mycorrhizal fungi (MF); dark septate endophytes (DSE); herbivory (HE); injury or bruising (IN); decay (DE); other (O); and none (N) were successful in surveying such extremely broad geographical and ecological categories. In future work, where specific pathologies are involved, the other (O) category can be expanded to include pathogen-specific lesions to facilitate identification and statistical analysis.

4.1.3 Lesion Characterization

When counting lesions, all associated structures were counted only once per field of vision, this was done to ensure that lesions with more than one characteristic structure (e.g., MF, DSE, DE, O) would not become over-represented or over-interpreted; e.g., if trypan-stained hyphae and vesicles were both present in a FOV, only one (1) count of MF would be scored.

Concerning histological artifacts, which will be discussed below, intracellular hyphae (e.g., arbuscules) could be easily confused with improperly prepared slides; it was therefore important to take into consideration the overall appearance and quality of the root sample and to look for evidence of any other associated fungal structures (e.g., vesicles, extra-cellular hyphae, etc.) before making a decision regarding the lesion tally.

Herbivory, bruising, and decay lesions are reflective of the physical health of the root system and can be interpreted as secondary effects of an infection, which the methodology cannot directly detect. The use of trypan blue dye (or any stain) is exceptionally useful in identifying

areas of roots which have been subjected to some types of trauma. The trypan dye does not penetrate intact cellular membranes, and as such areas of roots which have cells that have been compromised will uptake the stain with high contrast; additionally, it chemically binds to chitinin found in fungal cell walls as prepared in the methods section.

Across the various invasive plant species, observances of the other (O) category were sporadic, possibly due to removal during the processing stages.

Tweezer markings pose a risk of mis-identification with the herbivory lesion as they both typically present as semi-circular indentations; however, the tweezer artifacts do not display the typical bruising halo that herbivory does (figure 1).

4.1.4 Lesion Effects on Plant Biomass

In efforts to better characterize how soil microorganisms impact plant health, a 12-week indoor growth experiment was conducted. The idea of using two additional invasive species was used in-part to help demonstrate the robustness of the lesion counting protocol.

The positive association of biomass and soil sterility (figure 4) can be attributed in part to the abundance of nutrients and the absence of competitive effects from soil microbiota. Overall, this observation serves as an example of a competitive negative feedback between soil microbes and invasive plant species. The perspective provided by the none category in the survey methodology provides an objective estimate of total root colonization by soil microbes.

4.1.5 Lesion abundance and distribution based on treatment

It should be noted that because of the trypan blue staining process, any hyphae which contained chitin were stained blue and considered as MF. No attempts as species identification were made, and there is the possibility that not all counts are true MF species; however, this does not affect the application of the lesion count methodology as it is considered as an initial course of investigation.

It can be posited that no effect was observed for three reasons: i) 12 weeks is an insufficient amount of time for soil biota to adequately colonize root systems; ii) root systems have a high level of functional redundancy; iii) soil nutrients were so abundant that microorganisms didn't have to compete against each other or the plant. This is an important understanding to be carried forward into further research as the invasive species in which biomass is negatively affected by soil lesions could be indicative of a possible bio-control for use in other areas of its range; the same can be said for any overabundance of one type of lesion in a subpopulation as well.

4.1.6 Laboratory and Field differences

A 12-week experimental growth period was insufficient to establish a large amount of DSE colonization; this is an important observation as further laboratory or greenhouse research into lesion causes would require consideration into the growing conditions required for the lesion causing organisms themselves to grow. It is therefore recommended to use core samples in subsequent studies of lesions and their causes.

4.1.6 Native and exotic species

The experiment concerning the lesion-load in the native versus exotic species (figure 6) was important to demonstrate the counting methodology in direct comparison of exotic versus native plant species in a wild-type habitat. While the lesion categories followed the behavior of the group aggregate data in figure 2, the suppressed hyphae lesion count across native versus exotic for *B. inermis* and S. *lanceolatum* indicates a phenomenon for further identification and investigation, namely species-specific level effects and soil interactions.

4.1.7 Other considerations

Herbivory as a category is an important consideration and any species subjected to large amounts of individual herbivory indicates some import. The degree to which plants are herborized would be very important to know as it would let us know which species are susceptible to herbivory as a pathology. It is important to know which plants are being predated upon below ground, and which locations have predatory effects over others in terms of introduction and invasion ecologies and economics. Particularly in regards to invasive species there might be a way to identify which species in one region is predating the plant and develop biocontrol efforts in other areas through root lesion analysis.

The other category is a catch-all of miscellany intended to be able to account for any additional organisms or structures which may be affecting the health or structure of the plant. Other was included as a categorical for novelty and to keep the possibility of large regional variances and species variances contained, it was a difficult enough challenge to try to predict properly what would be observed with one species let alone 10 from 10 different locations across North America, this is a very generalized survey methodology.

A count of no observable lesions was maintained throughout the survey as lesions were often observed to overlap in the same field-of-vision. The ‘none’ category thus serves as a type of negative count allowing researchers a more binary source of root-soil interaction data of present-absent analysis. The ‘none’ category carries with it the theoretical implications that plant species with higher counts are ultimately more invasive, but this was not demonstrated in this initial survey.

4.1.8 Comment 2025

This document was written before the legalization and industrialization of cannabis/marijuana (CM) in Canada and at the State level in the United States. There is a particular social commentary during the time in which it was written: that CM was a plant that was industrially and professionally not tolerated and undesirable, even with the acknowledgement of the government controlled medical CM and regulated hemp industries. For many older individuals, societally: the CM plant and its products are viewed as an invasive plant species; with decades of: mental, social, and legal enforcement principles against CM as a nuisance species accurately paralleling invasive plant species management practices. In 2018, CM legislation was passed in both Canada (Federally) and the United States (on a per state basis) decriminalizing, legalizing, and industrializing CM and its products for recreational use by the public. While most CM is grown under controlled indoor/greenhouse conditions, this methodology to assess soil ecology through lesion-load identification can be used on the newly approved CM crops, and the standard agricultural crops to improve yields and develop hardier more disease-resistant cultivars.

There is a Europe-centric perspective in the original manuscript, but that should be modernized to just a North America-centric perspective because invasive species can and have been brought in from other continents of the world, not just Europe. Historically, even though it was the Europeans that attempted to colonize North America in large efforts, their actions brought plant species to North America from all corners of the planet, and vice versa.

5.1 Conclusions

This root lesion surveying methodology is a first-in-class, low-technology methodology for directly measuring and discovering new areas of investigation in soil ecology and invasive plant species invasion ecology and pathology. The experiments conducted using this new root-surveying methodology directly compares the presence and absence of seven (7) main categorical and ecologically important root-lesion phenomenon across soil sieve strata and invasive plant species, including assessing invasive versus non-invasive plant root lesions, and arboretum versus laboratory grown plant species. The surveying methodology is simple enough for the public to participate in citizen science, but statistically complicated enough for graduate students and post-doctoral researchers.

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